Health Effects from the Inhalation of Oxidant Air Pollutants as Related to the Immune System

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ABSTRACT

These investigations dealt with links between ozone inhalation and lung diseases. Increases in allergic lung disease occurred among our test populations of mice following inhalation of ozone at levels as low as 0.13 ppm, thus establishing a cause and effect relationship between the toxic inhalant and enhancement of a specific health problem.

When the ozone exposure was made intermittent by removing the 0.18 ppm of ozone for 9 hours each day during the 4 day ozone cycles, this also reduced the total ozone exposure to 60 hours per cycle rather than the previously used 96 hour cycles. Under these conditions there continued to be more allergically sensitized animals when compared to those maintained in ambient air.

An adjuvant substance was used to augment the immunological responses against the antigen (allergen). The adjuvant was inactivated Bordetella pertussis cells. This is the same material that is used to immunize against whooping cough in children. In mice receiving adjuvant, there was a significant enhancing effect for allergic sensitization from ozone at the level of 0.10 ppm. The adjuvant effect of whooping cough vaccine could still be detected in mice when they received only 1/49th or 1/24th of a dose used for childhood immunization. In this circumstance the environmental influence of periodic exposure to 0.15 or 0.18 ppm of ozone significantly increased the number of animals developing allergy.

Guinea pigs were used to see if they would show enhanced allergic sensitization from ozone exposure by a reaction similar to human asthma. This trial was not definitive and the factors involved would require additional investigation.

Ozone inhalation reduced the severity of pneumonia in mice from influenza virus infection. When infected lungs were examined for the presence of influenza virus, it was found that the lining cells of the airways were less affected by virus replication in animals that had inhaled 0.16 ppm of ozone. Both ozone inhalation and influenza virus infection cause edema fluid to collect in the lungs. In an experiment designed so that maximum edema from both causes would occur simultaneously, the effect persisted wherein fewer deaths occurred in ozone exposed animals than in infected mice maintained in ambient air.

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STANDARD DISCLAIMER

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SUMMARY

These investigations dealt with further clarification of links between ozone inhalation and lung diseases. Ozone has been shown by others to act as a powerful oxidizing agent producing histologically recognizable damage to the lining epithelial cells of the respiratory tract. Immunology offers real promise in determining events set in motion by air pollutants. Increases of immunological activity in the lung are clear indicators of injury to the tissues. Increases in allergic lung disease occurred among our test populations of mice following inhalation of ozone, thus establishing a cause and effect relationship between the toxic inhalant and enhancement of a specific health problem. Changes in the pattern of immunological defenses of the lung against infecting virus (influenza virus) followed exposure to ozone and offered evidence that the susceptibility of lung cells to the virus had been altered.

I. Studies on Allergic Enahancement from Ozone Inhalation in Mice

Mice were chosen as the experimental model animal for 3 reasons. 1) The immune system of mice has been more thoroughly investigated than that of any other species of animal. 2) Other investigators consider the mouse a valuable model animal for studying immunoglobulin E (IgE) antibody responses as it relates to man. Immunoglobulin E is the class of antibodies responsible for allergic reactivity. 3) The unit cost of mice is low enough to permit the use of statistically valid numbers of animals in comparison groups.

Six experiments were performed to analyze the enhancement of allergic sensitization by inhaled ozone. These were probing experiments to determine the threshold for this effect and, consequently, there was a stepwise decrease in the ozone concentrations employed. Significant allergic enhancement from ozone exposure was consistently demonstrated in concentrations ranging from 0.64 ppm down through 0.13 ppm. At the level of 0.10 ppm the enhancement effect from ozone disappeared.

Photochemical smog episodes are associated with diurnal variations in the ozone content of air. The cyclic variation is characterized by peak levels occurring in mid-afternoon and relatively low levels in the early morning hours. One trial was run to mimic this event by providing a rest period in ambient air during each ozone exposure day. The mice were held 9 hours in ambient air and 15 hours in 0.18 ppm of ozone. Once again there were more allergic animals in the ozone exposed group, but the magnitude of the difference was decreased when compared to animals maintained in ambient air.

II. Whooping Cough Vaccine as an Additional Factor Affecting Allergic Enhancement by Ozone

Another finding from our research has been the effect of an adjuvant on allergic sensitization. Adjuvants are substances injected along with antigen (allergen) to augment immune responses to the antigen. It was decided to shift the immune balance in animals toward synthesis of the IgE

class of antibodies. The bacterium, Bordetella pertussis, is known to contain a protein with the unique effect of modifying immune regulation to favor IgE synthesis. We gave the mice a single injection of inactivated B. pertussis cells. This bacterium causes whooping cough in children, and the injected material was the same as that used for immunization purposes. As previously explained the ozone enhancement effect disappeared at 0.10 ppm. However, when animals received the identical treatment in the 0.10 ppm experiment, but were also injected with B. pertussis cells, an augmentation of the IgE responses occurred and significant allergic enhancement was seen at the ozone concentration of 0.10 ppm. If the mice received doses of only 1/49th or 1/24 of the dose used for the immunization of children, it caused increases in allergic sensitivity among animals exposed to elevated ozone levels. The fractional dose referred to the total immunizing dose for children when the amount of vaccine for the mice was scaled down to a 30 gram body weight as compared to the body weight of an 8 pound child (3,629 grams). Thus, the combined effects from environmental exposure to ozone and the routine use of whooping cough vaccine may be working together, with a result leading to the establishment of respiratory allergies in children.

III. Study of Ozone Effects on an Asthma-like Animal Model

Guinea pigs were used to see if they would show enhanced allergic sensitization from ozone exposure by a reaction similar to human asthma. There was no evidence of an ozone effect in this single experiment. Additional research is warranted to discover if the ozone effects are variable among certain species or if future work proves to contradict this single result, with a relatively small test population.

IV. Effects on Influenza Virus-induced Pneumonia from the Inhalation of Ozone

Studies on influenza virus infection in mice have revealed that the disease process is less severe in animals that are held in a raised ozone environment when compared with animals maintained in ambient air. An experiment showed that the ozone effect was again seen at the reduced level of 0.16 ppm of ozone. When the infected lungs were examined for the presence of influenza virus, it was found that the lining cells of the airways were less affected by virus replication in animals that had inhaled 0.16 ppm of ozone.

Ozone inhalation and influenza virus infection both lead to the collection of edema fluid in the lungs. An experiment was devised wherein the maximum edema from both processes would occur simultaneously. In this circumstance it was once again found that the mortality rate was lower in animals that had inhaled ozone. It appears that there is less virus propagation in lining epithelium of the lungs when animals have experienced ozone oxidation of the respiratory tissues. This reduces severity of the disease and the mortality rate.

CONCLUSIONS

I. The threshold for allergic enhancement to an inhaled allergen by ozone exposure was found to lie between 0.10 and 0.13 ppm in the animal model. This ozone concentration is frequently present in the environment of man. When the ozone exposure was made intermittent, and reduced in amount, there continued to be more allergically sensitized animals when compared to those maintained in ambient air. The potential consequences for increasing the numbers of individuals with respiratory allergies poses a serious question for the prevention of human illness.

II. The adjuvant effect of whooping cough vaccine could be detected in mice when they received only 1/49th or 1/24th of a dose used for childhood immunization (DTP, Diptheria-Tetanus-Pertussis). In this circumstance the environmental influence of periodic exposure to 0.15 or 0.18 ppm of ozone significantly increased the number of animals developing allergy. Thus, the combined effects from environmental exposure to ozone and the potential use of whooping cough vaccine may be working together, and leading to increased numbers of respiratory allergies among children.

III. An inconclusive experiment was run to test asthma-like reactions in guinea pigs. The expected effect from ozone did not develop. Conclusions are not drawn from this single trial, with a relatively small test population, and further research is indicated.

IV. Ozone inhalation reduces the severity of pneumonia from influenza virus infection in mice. This effect persisted in an experiment designed so that maximum edema in the lungs from ozone exposure would coincide with maximum edema from the virus infection.

V. When infected lungs were examined for the presence of influenza virus it was found that the lining cells of the airways were less affected by virus replication in animals that had inhaled 0.16 ppm of ozone.

RECOMMENDATIONS

I. An adverse health effect from ozone exposure has been demonstrated as enhancement of lung sensitization to inhaled allergens in the mammalian lung. This should be brought to the attention of the medical community, and groups concerned with environmental protection. A carefully devised epidemiological study is now needed to examine the impact of this finding on human health. The use of this information for setting air quality standards needs consideration.

II. Experimental evidence is presented to show that the combined effects from ozone exposure and the use of whooping cough vaccine can increase the liklihood of establishing respiratory allergies. The potential health hazard arising from air pollution and a routine pediatric procedure requires serious investigation.

III. Although it is not imperative, it would be of interest to further investigate the use of an animal model to demonstrate allergic enhancement from ozone in an asthma-like response. Such a model demonstration would make this health effect from ozone more readily understood by the public.

IV. The mechanism by which inhaled ozone reduces the severity of influenza in mice is of considerable interest to the field of virology. Support for investigation of the matter would probably be incumbent on medical science research organizations.

PROJECT REPORT

Health Effects From The Inhalation of Oxidant Air Pollutants As Related To The Immune System

(A2-057-33)

I. Purpose and General Background of the Project

The appropriate stance to take regarding acceptable levels of air pollutants in California is affected by the increasing need to determine what actually happens to mammalian tissue following pollutant exposure. A growing body of epidemiological literature presents positive correlations of lung diseases and reduced pulmonary capacity with the presence of high levels of air pollutants (1-7). Skeptics may object to the interpretations of epidemiological investigations by questioning the control of variables, and by pressing for positive evidence of tissue changes. Controlled experimentation on animals offers a means for determining alterations in the immune responses of the respiratory tract as mediated by the presence of air pollutants. Ozone has been shown to act as a powerful oxidizing agent that produces histologically recognizable damage to the lining epithelial cells of the respiratory tract (8-12). The problems accruing from high ozone levels will persist in the foreseeable future as byproducts from the use of internal combustion engines.

Immunology offers real promise for determining events set in motion by air pollutants. Increases of immunological activity in the lung are clear indicators of injury to the tissues. Increases in allergic lung disease (asthma) among a test population of animals following inhalation of an air pollutant establishes a cause and effect relationship between the toxic inhalant and enhancement of a specific health problem. Changes in the pattern of immunological defenses of the lung against infecting virus (influenza virus) following exposure to ozone offers evidence that the susceptibility of lung cells to the virus has been altered.

The immune system is primarily concerned with the body's handling of foreign substances entering the tissues. If the foreign substance provokes an immune response, it is referred to as an antigen. For example, in an influenza attack the influenza virus acts as an antigen, and the immune responses to the viral antigen are specific antibodies and cells reacting with virus, which lead to its destruction and elimination from the body. A good functional immune system is, therefore, vital to the warding off of infections and the maintenance of a healthy state.

In some situations the immune responses play an undesirable role. An example is human asthma where the inhaled antigen is usually some inert substance, such as dog or cat dander, household dust, plant pollens, etc. The antibody response in this case is detrimental to health since the reaction of the antigen and antibodies in the lung leads to the release of body chemicals that contract the airways and produce the typical asthma attack. These varied responses of the lung to antigens are related to the multiple forms in which antibodies are made in the body. Four important classes of protein are now recognized as antibodies which perform different functions. The antibodies are called immunoglobulins (Ig), and the four classes are IgM, IgG, IgA, and IgE.

In the instance of asthma, the IgE antibodies are specifically responsible for the disease. Cells producing IgE are distributed along the respiratory mucous membrane.

II. Effect of Intermittent Ozone Exposure on Allergic Enhancement

A. Purpose and Background

We have demonstrated an adverse health effect from ozone exposure as enhancement of lung sensitization to an inhaled allergen in a mammalian animal model. Six experiments were performed to analyze the enhancement of allergic lung sensitization by inhaled ozone. These were probing experiments to determine the threshold for this effect.

Allergically sensitized mice were detected by the events of anaphylactic shock following injection of the allergen. An overview of results showing ozone concentrations, the numbers of mice used, and the probability values are reported in Table 1. The results were interpreted to mean that significant allergic enhancement from ozone exposure occurred in all ozone concentrations down through 0.13 ppm. At the level of 0.10 ppm, the enhancement effect from ozone disappeared.

Table 1 - Statistics on Allergic Sensitization of Mice Maintained in Filtered Ambient Air Versus Mice Exposed to Ozone[1]

Experiment	Ozone Level	Number of Mice	Probability	Significance
A	0.64 ppm	48	$P<0.018$	+
B	0.64 ppm	40	$P<0.007$	+
C	0.4 ppm	115	$P<0.001$	+
D	0.24 ppm	119	$P<0.0001$	+
E	0.16 ppm	183	$P<0.0195$	+
F	0.1 ppm	192	$P>0.5$	-

[1]In a given experiment the contact with aerosolized allergen was equal for the two compared groups. Ozone exposures were continuous for 3-4 day periods, which were repeated intermittently in 4 cycles over approximately 6 weeks.

B. Design - Materials and Methods

The circumstances of lung sensitization were based on findings from our previous studies. It was shown that damage to the respiratory membranes from ozone could be monitored by quaantitating the serum albumin levels in respiratory secretions (13). On day 4 of ozone exposure a peak of serum albumin in secretions indicated that this was a period of impaired membrane integrity, and a time when extrinsic antigen might easily gain access to immunocompetent cells in the tissues underlying the epithelium. The rationale for presenting airborne antigen after 3-4 days of continuous ozone exposure was based on the anticipation that sensitization might occur at that time. The basic design of these experiments is hown in Figure 1.

Figure 1

Ozone Effect on Allergic Enhancement in Mice:

Experimental Design for Ozone Exposure and Allergen Contact

	Days				Procedure			
1st, 2nd OA Aerosol	1	2	3	4	Ozone Exposure			
	5	6	7	8	9	10	11	Ambient Air
3rd OA Aerosol	12	13	14	15	Ozone Exposure			
	16	17	18	19	20	21		Ambient Air
4th OA Aerosol	22	23	24	25	Ozone Exposure			
	26	27	28	29	30	31	32	Ambient Air
5th OA Aerosol	33	34	35	36	Ozone Exposure			
	37	38	39	40	41	42	43	Ambient Air
	44	Shocking Injection of OA						

Ozone = Concentration was continuous over 4 day periods but varied in different experiments from 0.10 ppm to 0.64 ppm.

Allergen = Ovalbumin (OA); a 20 mg/ml solution was used for aerosolization and the shocking injections.

Mice (Specific Pathogen Free) were exposed to ozone in stainless steel chambers at the Environmental Chamber Facilities of the California Primate Research Center. Ozone concentration was continuously monitored by the ultraviolet photometric ozone analyzer (Dasibi Environmental Corporation, Glendale, CA). Control animals were housed in an environment of filtered ambient air.

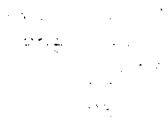
Mice were allergically sensitized to an allergen by inhalation of an aerosolized allergen (lung sensitization). The allergen was ovalbumin (OA), which is a purified crystalline form of albumin obtained from the chicken egg. Ovalbumin was used to mimic the inhalation of environmental allergens, such as plant pollen. A variety of proteins from plant, animal or microbial sources can be allergens in man.

Ovalbumin served well as an experimental allergen since its size of 44,000 daltons placed it in that range of proteins that are small enough to be absorbed through mucous membranes and large enough to be sufficiently complex to function as immunogens [14]. The contact between environmental allergens and susceptible individuals can be prolonged and nearly continuous (i.e., the pollen of plants, or the presence of animal dander from pets). However, allergen contact was necessarily limited in the experiments, and the times of contact were estimated to be periods when the antigenic stimulation would be effective.

After 4 days of continuous ozone exposure the mice had allergen contact from nebulized OA in a Tri-R Airborne Infection Apparatus (Tri-R Instruments, Inc., NY). The nebulizer was designed to yield aerosol droplets of less than 3 μm (range of 0.5 to 3 μm). Mice were placed in the chamber and were exposed for 30 min to nebulized OA (2% solution in sterile distilled water). The relative humidity in the chamber was approximately 50% at 22°C.

The animals were then maintained in ambient air for several days before the cycle of ozone and aerosolized antigen was repeated over 4 allergen contact cycles. Times when the animals were held in ambient air and then returned to the ozone chambers were to simulate the intermittent phasing of low and then high air pollution episodes that may occur in the environment. Animals were rested in ambient air for a week after the last allergen contact and were then tested for allergic sensitization.

A brief account of events in this type of allergy includes the following considerations. IgE is well established as the antibody responsible for sensitizing individuals for asthmatic attacks and anaphylactic shock [15]. The B-lymphocytes synthesizing IgE are located in bronchus-associated lymphoid tissue of the lung under the epithelial lining of the airways [16]. Antigenic stimulation of these cells follows the inhalation of allergens which gain access to the subepithelial spaces. This leads to the synthesis of IgE with specificity for the allergen. The IgE molecules fix to mast cells by receptors on the antibody molecules. Such a mast cell is sensitized. If the allergen is reintroduced, as by inhalation, the resulting antigen-antibody reaction causes the mast cell to degranulate and release pharmacologically active substances that produce the tissue responses seen in an asthma attack. An individual experiencing asthma from inhalation of the allergen can also experience anaphylactic shock if the allergen is injected in sufficient quantity. Thus, shock is associated with generalized anaphylaxis, and asthma is a syndrome of local anaphylaxis.



Since the lung is not a dominant shock organ in allergically sensitized mice, it was necessary to reveal allergic sensitization by another means. The test procedure chosen was induction of anaphylactic shock following the intravenous injection of OA. In sensitized animals the signs of increased respiration were apparent 1 to 2 min after the injection of antigen. Cyanosis was obvious as darkening of the eye and ear color, and the mice became increasingly listless and then prostrate. Within 10 min most of the severely sensitized animals were prostrate. Deaths occurred 20 to 40 min after the injections. The mice were watched carefully over a 2-hr period, and those destined to survive the anaphylactic shock gradually increased their activity as the syndrome subsided. A few survivors gained a normal appearance within 40 min, but some severely affected individuals remained cyanotic and immobile, with ruffled fur, for more than 2 hours.

Each experiment required that several animal groups be tested for anaphylactic sensitivity. The positive-control group had been sensitized by two injections of ovalbumin, and the development of shock in nearly all members of this group demonstrated that a functional allergic disease state had been established. Normal mice were injected as the negative-control group, and their failure to display any ill effects from the ovalbumin injection showed that the injected material was free from inherent toxicity. Important groups were those that had simply been exposed to aerosolized antigen, since some members of those test populations would, as expected, become allergically sensitized. Comparison of these filtered ambient air control groups with animals that had additionally been exposed to ozone was the critical test for revealing enhancement of the allergic state by ozone.

Photochemical smog episodes are associated with diurnal variations in the ozone content of air. The cyclic variation is characterized by peak levels occurring in mid-afternoon and relatively low levels in the early morning hours. One trial was run to mimic this event by providing a 9 hr rest period in ambient air during each ozone exposure day. The circumstances of lung sensitization were based on findings from our previous studies. The basic design of the experiment was the same as that which has been described. However, in this trial each day of ozone exposure allowed the animals a rest in ambient air for 9 hours during the work day and a 15 hour exposure to 0.18 ppm of ozone overnight. These 4-day cycles of intermittent ozone exposure were repeated 4 times. The mice were sensitized by inhalation of aerosolized OA in the usual way and the same set of test system controls was included.

C. Results and Discussion

In this trial, one animal among 97 maintained in filtered ambient air showed anaphylactic shock symptoms. In the group exposed to ozone, 4 out of 96 experienced anaphylaxis, and 2 of these cases were fatal. The chi-square test indicated the probability of obtaining this result by chance as approximately 1 in 7 times.

The trend of the data from this experiment was in accord with our previous studies, which showed more allergic sensitization in test animals exposed to ozone. However, the magnitude of the difference in response was decreased.

The result from this single experiment with intermittent exposure to ozone suggested reduced likelihood of establishing allergic lung sensitization as compared to continuous exposure to the oxidant. In this experiment the ozone exposre was reduced, since the elevated ozone was removed for 9 hours each day during the 4 day ozone cycles. Thus, the total ozone exposure per cycle was 60 hours, rather than the previously used continuous exposure for 96 hours. The decreased ozone exposure could have diminished the membrane damage, or perhaps the repeated 9 hr rest periods from ozone exposure allowed for some level of membrane repair or adjustment to reduce the opportunity for antigenic stimulation from inhaled allergen. This could be an important factor in reducing the potential for development of respiratory allergies in the human population when subjected to elevated ozone levels. It may be a fortuitous event that photochemical smog has high and low cycles during each 24 hr period.

However, this is not to suggest that diurnal cycling in the concentration of photochemical smog eliminates the effect of ozone for allergic lung enhancement. In the intermittent ozone trial, the degree of allergic enhancement by ozone was reduced, but it is prudent to recognize that allergic effects were, once again, more severe in the ozone exposed animals. Further experimentation will be necessary to fully understand effects arising from daily fluctuations in ozone levels. Another factor to consider is the effect from daily periods of high ozone concentration during photochemical smog episodes. In our animal experiments, the periods of ozone exposure were kept uniform at a planned concentration. However, in an example smog period (month of September, 1980, in the California Southcoast Air Basin) there were 17 days when the hourly average ozone concentration exceeded 0.21 ppm. During those 17 days, there were 54 hours when the average ozone levels exceeded 0.21 ppm with concentrations ranging up to an hourly average of 0.49 ppm. It would be expected that these periods of high ozone exposure would be more deleterious to the respiratory membranes than uniform levels at lower concentrations.

III. Whooping Cough Vaccine (Bordetella pertussis) as an Additional Factor Affecting Allergic Enahncement by Ozone

A. Purpose and Background

When experiments were performed at low ozone concentrations, it was expected that the ozone enhancing effect would diminish. To determine whether anything of significance was occurring from the presence of ozone it was decided to shift the immune balance in the animals toward synthesis of IgE. The bacterium, Bordetella pertussis, is known to contain a protein with the unique effect of modifying immune regulation to favor IgE synthesis (17). We gave the mice a single injection of inactivated B. pertussis cells. This bacterium causes whooping cough in children and the injected material was the same as that used for immunization purposes. It has already been explained that ozone enhancement occurred at 0.16 ppm but that the effect disappeared at 0.10 ppm. However, when animals received the identical treatment but were injected with B. pertussis cells in addition, an augmentation of the IgE responses occurred. The important finding occurred in animals exposed to ozone, which showed significant allergic enhancement at the ozone concentration of 0.10 ppm ($P=<0.001$). Therefore, when the IgE responsiveness in individuals was increased, the threshold ozone concentration for producing an effect was reduced.

B. Design - Materials and Methods

It is routine practice to inject children from a few months of age until the sixth or seventh year, with inactivated Bordetella pertussis cells, or fractions of the cells, to induce resistance against the agent of whooping cough. Five doses of the vaccine are injected during this period of life. Usually the vaccine also contains diphtheria toxoid and tetanus toxoid to induce protection against the potent toxins of diphtheria and tetanus. The DTP vaccine (diphtheria, tetanus, and pertussis) is combined as a triple antigenic preparation.

In two of our previous experiments, using the mouse model, we have found that injected Bordetella pertussis cells increased the numbers of sensitized animals when the mice were allowed to inhale an allergen. In addition, the presence of 0.16 ppm and 0.10 ppm of ozone were shown to significantly increase the numbers of sensitized animals when compared with mice maintained in ambient air.

It was of interest to know whether the effect from the vaccine would persist if the mice received a smaller dose of the pertussis adjuvant than had been used in the described experiments. The factors of body weight and age were considered to make the study more nearly approximate the human experience. The dose for an 8-pound infant was scaled down as the equivalent amount for a 30-gram mouse. Mice were immunized at six weeks of age, which would approximate the fourth year of age for a child.

The basic design of the experimental protocols was the same as described previously for ozone enhancement of allergic sensitivity. Each experiment included eight major test groups. Four groups were exposed periodically to 0.15 ppm of ozone; while four additional groups were maintained in ambient air. Each set of four groups included a group receiving no pertussis adjuvant, a group receiving a human equivalent dose of pertussis adjuvant, a group receiving 2.5 times the human equivalent dose, and a group receiving 5 times the human equivalent dose. The adjuvant effect from a commercially available DTP vaccine for the immunization of children was used for the test (Wyeth Labs. Inc. Marietta, PA).

Results from the initial experiment gave rise to uncertainty as to whether the experimental mice (Hilltop Lab Animals, Scottdale, PA) were showing the expected level of immunoresponsiveness. Consequently, the experiment was repeated using mice from another supplier (Charles River, Portage Bay, MI) and ozone at the level of 0.18 ppm. Further information on the experimental protocol is detailed as follows:

Air Pollution Experiment #56
(AP-56)

Objectives:

1. To determine the adjuvant effect of commercial DTP vaccine for IgE synthesis (Wyeth-aluminum phosphate adsorbed).

2. DTP vaccine to be tested at three dose levels:
   I. A dose level comparable to the human dose based on body weight (30 gm mouse vs. an 8 pound child [3,629 gm]).
   II. A dose 2.5 times dose I.
   III. A dose 5 times dose I.

3. To determine the allergic enhancing effects of ozone (0.18 ppm) in the presence of the three levels of BP adjuvant, and in the absence of adjuvant.

4. Mice to be sensitized and shocked with the same concentration of OA used in previous mouse studies (20 mg/ml).

Animals:

5-6-week-old female SPF Swiss-Webster mice from Charles River in Portage Bay, MI.

Exposure Chambers:

Mice will be housed in stainless steel chambers as follows:

Ozone (0.18 ppm) - 60 mice w/BP - Dose Level I
60 mice w/BP - Dose Level II
60 mice w/BP - Dose Level III
60 mice w/o BP
10 mice to receive ozone but no aerosolized OA
10 mice to receive ozone and 2 BP injections (Dose Level III) but no aerosolized OA

Ambient Air - 60 mice w/BP - Dose Level I
60 mice w/BP - Dose Level II
60 mice w/BP - Dose Level III
60 mice w/o BP
10 mice normal (unmanipulated)
10 mice normal, except for 2 BP injections (Dose Level III)
10 mice - positive controls - 2 OA injections
10 mice - positive controls - 2 OA & 2 BP injections (Dose Level III)

Procedure:

1. The vaccine-injected mice are to receive an intramuscular injection on day 3 and day 25. Use the hind limb and alternate the limb for each injection.
2. Wyeth vaccine to be diluted as follows for each immunization day:

Dose Level	Dilution	Volumes	Units/0.1 ml
I	1:12.1	1.5 ml vaccine + 16.65 ml diluent= 18.15 ml total	0.0662 units
II	1:4.84	3.5 ml vaccine + 13.44 ml diluent= 16.9 ml total	0.165 units
III	1:2.42	7 ml vaccine + 9.94 ml diluent= 16.94 ml total	0.331 units

C. Results and Discussion

The results from these two experiments were similar and responses of the animal groups are summarized on Table 2. It was apparent that pertussis vaccine at dose level 1 had no effect for enhancing allergic responses. On the basis of body weight, this dose was estimated to be a mouse equivalent dose to that received by children undergoing immunization. The dose given to mice was only 1/121st of that injected into children. When the dose was increased by either 2.5 times or 5 times, the groups of vaccinated mice were uniformly more allergic if exposed periodically to elevated levels of ozone (dose levels II and III). When calculated within a given experiment, and at a single dose level, the difference in response between animals exposed to ozone and those maintained in ambient air was not significant. However, the trend of results in these animal groups all accumlated in the same manner; and when aggregated, the ozone exposed mice were calculated to experience a significant increase in allergic sensitivity over those maintained in ambient air ($P=<0.05$). Thus allergy was enhanced among mice exposed periodically to 0.15 or 0.18 ppm of ozone if they had received whooping cough vaccine in doses that were 1/49th or 1/24th of the dose used for the immunization of children. The fractional dose refers to the toal dose for both children and mice. The total dose was divided into two injections for the mice and five injections are recommended for immunizing children. On the basis of life span data the mice injected at six weeks of age were comparable to children in the fourth year of age.

The significance of ozone inhalation for enhancement of respiratory allergies in children, and the added augmentation that may come as a result of immunization against whooping cough are questions that require further investigation. Proponents for the vaccine may wish to assume that the amount of B. pertussis injected would be too small to have an adjuvant effect in

the children for stimulating IgE synthesis. The mouse experiments were performed here to determine whether very small doses of a commercially available whooping cough vaccine would reveal adjuvant action for IgE synthesis in mice. Clear evidence of adjuvant effect was obtained in our previously reported experiments when mice were exposed to 0.16 to 0.10 ppm of ozone. In those studies a dose of 5 optical units of a commercial vaccine (Connaught Labs. Ltd., Willowdale, Ontario, Canada) was injected into each mouse. That was 1/12th the amount used for the immunization of a child (60 optical units). However, on the basis of body weight the mouse dose was also about 12 times the amount that a child would receive. The threshold amount of the protein from B. pertussis that will function to shift the balance of immune response toward the production of IgE is not known. Perhaps the use of comparative body weights between species is not actually a valid basis for determining a threshold adjuvant dose. Nevertheless, in the absence of more precise information, these mouse experiments were devised by scaling the dose of vaccine for an 8 pound baby down to the body weight of a 30 gram mouse.

Table 2 - Effects of Ozone and Whooping Cough Vaccine on Allergic Sensitization of Mice

Whooping Cough Vaccine			Environment	Experiment A	Experiment B
Dose Level	Units/ Mouse	Fraction of Human Dose		0.15 ppm O_3	0.18 ppm O_3
I	0.132	1/121	Ambient Air	0/53*	3/60
I	0.132	1/121	Ozone	0/58	2/60
II	0.330	1/49	Ambient Air	0/56	1/59
III	0.662	1/24	Ambient Air	7/56	8/60'
Total mice sensitized in ambient air at dose levels II & III = 16/231					
II	0.330	1/49	Ozone	4/60	4/60
III	0.662	1/24	Ozone	10/60	12/60

Total mice sensitized in ozone at dose levels II & III = 30/240

* Mice allergic/Mice tested.

The adjuvant effect of whooping cough vaccine could be detected in the mice when they received only 1/49th or 1/24th of a dose used for childhood immunization. In this circumstance the environmental influence of periodic exposure to 0.15 or 0.18 ppm of ozone significantly increased the number of animals developing allergy in the test population. Evidence that the whooping cough vaccine has an adjuvant effect favoring IgE synthesis was shown in a medical report (18). Children receiving the usual DTP vaccine mixture were shown to develop allergic sensitivity to the tetanus toxoid present in the vaccine. Subsequent injection of tetanus toxoid would then

elicit anaphylactic shock symptoms. This information suggested that the current whooping cough vaccine can function as an adjuvant to increase the likelihood of inducing allergies when the body is stimulated by potential allergens. Thus, the combined effects from environmental exposure to ozone and the routine use of whooping cough vaccine may be working together, with a result leading to the establishment of respiratory allergies in children. This potential health hazard arising from air pollution and a routine pediatric procedure on children requires serious investigation.

IV. Study of Ozone Effects on an Asthma-like Animal Model

A. Purpose and Background

An objective of the project was to elicit allergic enhancement by ozone in an animal model where the reaction would be an analogue of an asthma attack in human subjects.

The lungs of guinea pigs respond to allergic reactions with symptoms resembling those of the human lung during an asthma attack. A central feature of this is contraction of bronchiolar-smooth muscle and the accompanying effect of airway restriction (19). When sensitized guinea pigs inhale an allergen, they appear uneasy and show labored breathing. In severe attacks, they may collapse and even die through lack of air (anoxia).

It seemed appropriate to demonstrate allergic responsiveness of lung tissue in an animal model and to see if the model would show enhanced allergic sensitization from ozone exposure.

B. Design - Materials and Methods

Initially, preliminary trials were performed to determine appropriate allergen exposure protocols. In order that the effect of ozone might be observed, it was first necessary to establish a regimen for allergic sensitization of guinea pigs breathing ambient air. A low level of allergic reactivity was needed in that animal group so that any augmentation of the response in an ozone exposed group could be compared.

The first two trials were performed on guinea pigs maintained in ambient air to gauge the sensitizing dose required to achieve the desired level of response. A third trial was performed wherein the dose of aerosolized allergen was used at the concentration of a 0.1% solution. Four guinea pigs were maintained in ambient air. Four additional guines pigs were held in a chamber containing 0.2 ppm of ozone for 4 days. They were then exposed to aerosolized allergen along with the ambient air control guinea pigs. All animals were held in ambient air for a week, then the cycle was repeated for a total of three 30-minute sensitizing exposures to the allergen. The animals were again rested a week in ambient air. All of the guinea pigs were then tested for asthma-like responses by inhaling a 2% solution of aerosolized ovalbumin. Table 3 presents results from this trial.

Table 3 - Preliminary Trial for Observing Asthma-like Responses in Ozone Exposed Guinea Pigs

Environment	Guinea Pig	Symptoms from third allergen contact*	Symptoms from provoking dose of allergen**	Test for skin sensitizing antibodies
Ozone Exposed	#1	None	None	Negative
Ozone Exposed	#2	None	None	Negative
Ozone Exposed	#3	None	None	Weak positive
Ozone Exposed	#4	Dyspnea	Dyspnea and collapse	Strong positive
Ambient Air	#1	None	None	Negative
Ambient Air	#2	None	None	Negative
Ambient Air	#3	None	Dyspnea and collapse	Strong positive
Ambient Air	#4	None	None	Negative

*Aerosolized ovalbumin solution (0.1%).
**Aerosolized ovalbumin solution (2.0%).

Information from a third trial was used to design an experiment using the following animal groups:

20 guinea pigs - exposed periodically to 0.2 ppm of ozone for 4 continuous days, and allowed to inhale an aerosol containing 1 mg/ml of ovalbumin (allergen).

20 guinea pigs - maintained in filtered ambient air and allowed to inhale an aerosol containing 1 mg/ml of ovalbumin (allergen).

4 guinea pigs - control animals exposed to ozone, but no sensitization with the allergen.

4 guinea pigs - control animals maintained in filtered ambient air.

Figure 2 shows the times for ozone exposure, allergen aerosolization for sensitization, and presentation of the provoking allergen aerosol.

Figure 2
Schedule for Sensitization of Guinea Pigs
with Aerosolized Ovalbumin

Sensitizing Ovalbumin Aerosol	Days				Procedure			
1st OA Aerosol	1	2	3	4				Ozone Exposure
	5	6	7	8	9	10	11	Ambient Air
2nd OA Aerosol	12	13	14	15				Ozone Exposure
	16	17	18	10	20	21	22	Ambient Air
3rd OA Aerosol	23	24	25	26				Ozone Exposure
	27	28	29	30	31	32	33	Ambient Air
	34	Provoking Ovalbumin Aerosol						

Sensitizing Antigen = 1 mg/ml Ovalbumin
Provoking Antigen = 20 mg/ml Ovalbumin
Ozone Concentration = 0.2 ppm

Allergic lung responses were evidenced as airway restriction. This was observed by an increasing order of severity as dyspnea, collapse, or death. Dyspnea refers to difficult breathing. Specifically, in this circumstance there was expiratory dyspnea since airway restriction hindered free movement of air from lungs. To score an animal as either normal or dyspneic was the only observation recorded that might be considered subjective, and we were especially interested in determining the validity of our scores on this observation.

At the time of the second aerosol, one ozone exposed animal showed slight dyspnea. When the third sensitizing aerosolization of allergen was delivered, we were alarmed to find that all of the animals in the 2 major test groups showed signs of dyspnea. The allergic responses were proving to be more extensive than had been predicted from results of the third pre-trial study. A search for an explanation revealed that the flow characteristics of the aerosolization machine had been slightly altered at a time when the instrument was moved to a new location. As a result, more allergen was delivered per unit time. The consequence of this was apparent when the provoking dose of allergen was delivered as the fourth contact of the animals with the allergen. All of the test animals had become allergic with results obtained as depicted in Table 4.

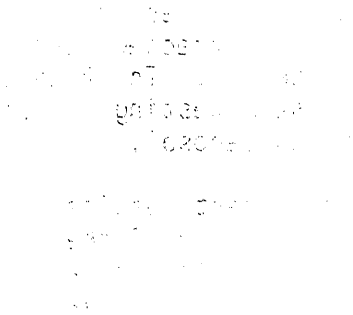


Table 4 - Observed effects from allergen inhalation in guinea pigs

No. of Animals Demonstrating:	Guinea Pigs Exposed to 0.2 ppm of Ozone	Guinea Pigs Maintained in Ambient Air
Dyspnea	20	20
Collapse	13	12
Death	2	5

One objective of the experiment had been to determine whether 0.2 ppm of ozone would enhance allergic lung sensitization. Unfortunately, that question was not answered because the level of sensitization had reached 100% in the group held in filtered ambient air. It was necessary to repeat this study with sensitization adjustments made to obtain effects similar to those of the third pre-trial.

Several other objectives of the experiment were achieved. It was shown that allergic reactivity in the guinea pig lung was a sensitive model for studying factors affecting allergic responses. Animals responded to the inhaled antigen with the formation of skin sensitizing antibodies. This was detected by skin testing the animals 2 days after the last aerosolization of ovalbumin. Intradermal injection of ovalbumin gave positive wheal and flare reactions. Tests were read 30 minutes after allergen injection, and the diameters of skin reaction areas were measured. When 5 μg of ovalbumin were injected intradermally, the immune reaction gave rise to skin swellings that were approximately 1 cm in diameter. These injections were controlled by the injection of equal volumes of physiological saline solution, which usually left the skin unaffected, but produced barely detectable swelling in 5 animals out of 33 tested. The 2 sets of control guinea pigs (8 animals) gave no reaction on skin testing and showed no symptoms when presented with the provoking allergen aerosol.

The positive skin test results in sensitized animals offered important confirmation on the validity of the observations on dyspnea as evidence of airway restriction when allergen was inhaled. Skin tests were uniformly positive among 15 animals that developed dyspnea and then subsequently recovered. Such animals were appropriately scored as allergically sensitized on the basis of the dyspneic symptoms.

Strong allergic reactions produced airway restriction to inhaled allergen that could be fatal. Necropsy examination of animals developing such reactions showed that air had been trapped in the lungs, which were greatly expanded when compared to the collapsed lungs of normal specimens.

The allergic reaction was also studied histologically. Lungs from non-allergic animals had collapsed alveolar spaces with normal cellularity and structure in the gas exchange tissues. The alveolar spaces were distended from trapped air in reacting lungs, and in many instances the delicate alveolar walls were torn and displaced.

Altnough the guinea pigs proved to be highly responsive to the effects of allergen-induced airway restriction, it was apparent that there could be some variation in the ease with which they became sensitized. Finding the correct experimental protocol proved to be quite difficult. Four additional pre-trials were made as background for the experiment which is presented in the Results and Discussion section.

C. Results and Discussion

A study was performed on guinea pigs to determine allergic lung activity from inhaled allergen. The animals were housed in stainless steel environmental chambers, and divided into subgroups as follows:

Ozone (0.25 ppm) Chamber:

12 g. pigs for 0.010 mg/ml allergen - 6 g. pigs - 3 sensitizations
6 g. pigs - 4 sensitizations

12 g. pigs for 0.015 mg/ml allergen - 6 g. pigs - 3 sensitizations
6 g. pigs - 4 sensitizations

4 g. pigs (ozone controls - no allergen contact)

Ambient Air Chamber:

12 g. pigs for 0.010 mg/ml allergen - 6 g. pigs - 3 sensitizations
6 g. pigs - 4 sensitizations

12 g. pigs for 0.015 mg/ml allergen - 6 g. pigs - 3 sensitizations
6 g. pigs - 4 sensitizations

4 g. pigs (normal controls - no allergen contact)

Aerosolized albumin was used as the allergen and guinea pigs were sensitized by allergen inhalation as presented in the diagram (Figure 3).

Figure 3
Schedule for Ozone Exposure and Sensitization
of Guinea Pigs with Aerosolized Ovalbumin

Sensitizing Ovalbumin Aerosol	Days				Procedure			
	1	2	3	4				Ozone Exposure
1st OA Aerosol	5	6	7	8	9	10		Ambient Air
	11	12	13	14				Ozone Exposure
2nd OA Aerosol	15	16	17	18	19	20	21	Ambient Air
	22	23	24	25				Ozone Exposure
3rd OA Aerosol	26	27	28	29	30	31		Ambient Air
1st Provoking Aerosol	32	33	34	35				Ozone Exposure
4th OA Aerosol	36	37	38	39	40	41		Ambient Air
	42							Second Provoking Aerosol

Sensitizing Allergen = 0.010 mg/ml or 0.015 mg/ml Ovalbumin
Provoking Antigen = 20 mg/ml Ovalbumin
Ozone Concentration = 0.25 ppm

Each aerosolization was preceded by a four-day period of continuous exposure at 0.25 ppm of ozone for half of the animals. The same aerosolization schedule was followed for guinea pigs held in filtered ambient air throughout the trial. Sensitization was tested by the inhalation of concentrated allergen, which was referred to as the provoking aerosol.

The experimental protocol was similar to that used with the mouse model. Aerosolized allergen (OA) was presented as a 0.01 mg/ml or a 0.15 mg/ml solution of OA in a series of 3 or 4 sensitizing contacts. The allergen contacts were timed to occur at the end of 4 day continuous exposure periods to ozone (0.25 ppm). The reaction provoking aerosol of ovalbumin, in a concentration of 20 mg/ml, was given 6 days after the last sensitization. The guinea pigs were easily rendered allergic from inhalation of ovalbumin. The concentrated test aerosol of ovalbumin induced

fatal bronchoconstriction in some animals and a milder reaction of dyspnea in others. Animals recovering from dyspnea produced wheal and flare skin reactions when 5 μg of ovalbumin was injected intradermally 2 days later. However, the trial did not suggest that ozone enhanced allergic reactivity in this species since 11 or 24 guinea pigs maintained in filtered ambient air developed allergic responses, while 8 of 24 ozone exposed guinea pigs reacted (Table 5).

Table 5 - Sensitization of Guinea Pigs to Aerosolized Allergen* when Exposed to an Elevated Ozone Environment (0.25 ppm) or Filtered Ambient Air.

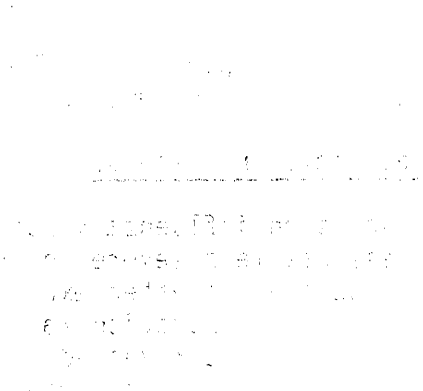
Response to provoking** aerosol of ovalbumin	3 sensitizations with 0.01 mg/ml of OA		3 sensitizations with 0.015 mg/ml of OA	
	Ozone Exposure	Ambient Air	Ozone Exposure	Ambient Air
Dyspnea***	1	0	2	1
Fatal reaction	0	1	0	1
Number sensitized	1/6†	1/6	2/6	2/6
	4 sensitizations with 0.01 mg/ml of OA		4 sensitizations with 0.015 mg/ml of OA	
Dyspnea***	2	4	2	1
Fatal reaction	1	1	0	2
Number sensitized	3/6	5/6	2/6	3/6
Total sensitized	4/12	6/12	4/12	5/12

* Allergen = Ovalbumin (OA).
** Allergic responses provoked by an aerosol containing 20 mg/ml of OA.
*** Sensitization confirmed by reaction to intradermal injection of 0.005 mg OA.
† Number allergic/number tested.

Combined Result

	Ozone Exposed		Ambient Air	
Number sensitized	8/24	33.3%	11/24	46%

In this single experiment with the guinea pig model, there was no evidence of an ozone effect. This was an unexpected result, since research by others had indicated respiratory membrane changes from ozone inhalation that were similar to those seen in mice and rats (20). Conclusions regarding the use of the guinea pig model should not be made from this single study on a relatively small test population. Rather, additional research is warranted to discover if the ozone effects are variable among certain species or if future work is found to contradict the result reported here.



V. Effects on Influenza Virus-induced Pneumonia from the Inhalation of Ozone

A. Purpose and Background

It is of special interest that the influenza virus affects many of the same cells in the respiratory system that are damaged by ozone inhalation. We have investigated the pathogenesis of influenza virus infection in mice by exposing them to aerosols of influenza virus, and examining the infected respiratory tissues.

Previous work showed that fatal influenza infections were less frequent in ozone exposed animals (0.40 ppm and 0.64 ppm) than in infected mice that were breathing ambient air. This surprising observation was made in 4 separate experiments. Two factors appeared to interplay in producing this effect. First, abnormal respiratory membranes developed as a result of 15 days of continuous ozone exposure, and this appeared to change the role of lining epithelium as host cells for influenza virus. Second, ozone at the concentration used was found to inactivate influenza virus. This effect could diminish the spread of virus within the airways. Broad interpretations could not be made on the basis of these virus infection results because of the ozone concentrations used, and the continuously maintained elevation of ozone was not in accord with usual environmental conditions.

In the previous project period an experiment was run at the reduced ozone concentration of 0.16 ppm to approach a more typical condition and to minimize the opportunity for ozone inactivation of virus in the lung.

Mice were maintained for 14 days of continuous exposure to 0.16 ppm of ozone in chambers as previously described. An equal number of animals was held in a chamber receiving filtered ambient air. The mice were then exposed to nebulized influenza virus (Influenza A_0 virus [WSN] propagated in Madin-Darby bovine kidney cells). The virus aerosol was generated in the TRI-R Airborne Infection Apparatus. The animals were held for an additional 14 days in either the ozone or ambient air environment.

B. Design - Materials and Methods

Previous studies on influenza virus infection in mice had revealed that the disease process was less severe in animals that were held in a raised ozone environment before and after exposure to the virus. In those experiments the ozone concentration was quite high (0.64 and 0.40 ppm). Our *in vitro* investigations have revealed that ozone at those levels can readily inactivate influenza virus. Ozone reduced to a level of 0.16 ppm did not adversely affect the virus during the first 8 hours of contact, and it was considered necessary to perform an experiment at this reduced level to determine whether the mortality-sparing effect of ozone would disappear. The experiment has been run and mortality data were obtained as shown on Table 6.

Table 6 - Mortality Pattern of Influenza Virus Infection in Ozone Exposed in Mice

	Day of Death Following Infection								
Animal Group	7	8	9	10	11	12	13	14	Total
Filtered ambient air	0	4	8	2	2	1	2	2	21
Ozone exposed(0.16 ppm)	0	0	3	1	1	0	0	0	5

Death occurred in 5 animals out of 60 in the ozone group and 21 animals out of 60 in the ambient air group. Chi-square analysis indicated a significant difference in the animal responses with a probability of <0.001. An effect from ozone was seen again, even at this reduced concentration of the oxidant. Lung tissues were analyzed from this experiment to determine tissue sites where influenza virus was concentrated.

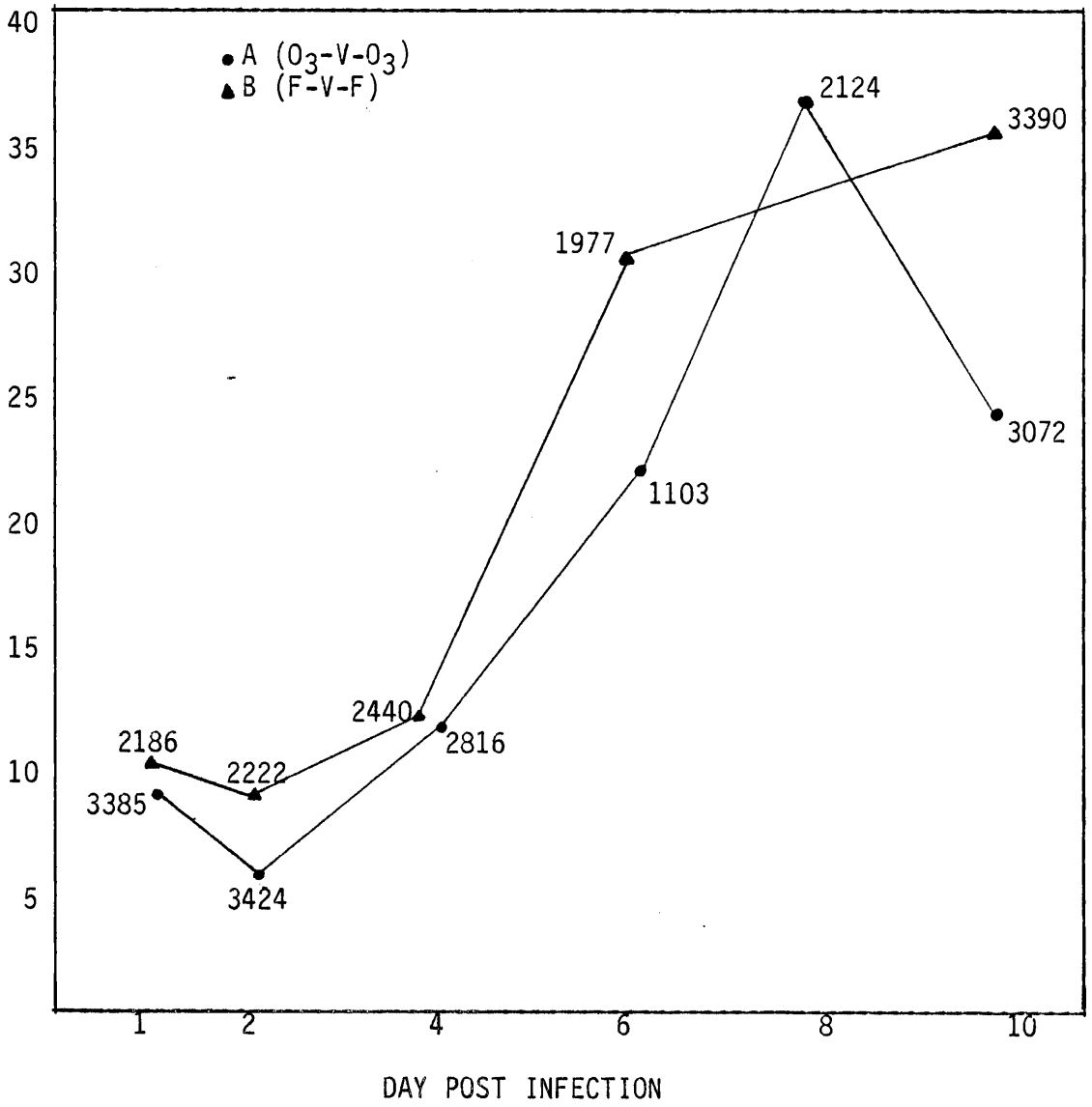
In previous work we have analyzed lung lavages for their content of serum albumin. Since serum albumin is synthesized in the liver and released into the blood, this protein can be present only in respiratory secretions as a filtrate of serum. In disease states, when vascular permeability is increased and membrane integrity is decreased, the albumin levels rise in the respiratory secretions, as a filtrate of serum. When animals are exposed to ozone, the albumin rises and reaches a peak on the 4th day of exposure. During an influenza attack the inflammation in the major airways and lungs is associated with extensive accumulations of edema fluid. In the experimental mice we find that this reaches a peak on the 8th day of the infection, and that fatalities accumulate over the following days. It is apparent that edema is a result of both ozone exposure and influenza virus infection. The severity of the disease process could be increased if the two edema effects occurred simultaneously, and such circumstances could, of course, easily occur in the natural situation.

An experiment was performed wherein mice were infected with aerosolized virus, and after 4 days into the virus process they were exposed to 0.16 ppm of ozone continuously for 14 days. The results were compared with mice that were simultaneously infected with virus and maintained in ambient air.

C. Results and Discussion

1. Effect of Reduced Ozone Concentration (0.16 ppm) on the influenza Process. Studies on influenza virus infection in mice have revealed that the disease process is less severe in animals that are held in a raised ozone environment, before and after exposure to the virus. An experiment showed that the ozone effect was again seen at the 0.16 ppm level, as had been described in previous progress reports. Tissues from these mice were studied to determine the location of influenza virus in the lungs. The technique of immunofluorescence was used with fluorescein labeled anti-fluenza virus antibodies which bind to virus in tissues, and are then examined by means of the fluorescence microscope.

Figure 4
Distribution of Influenza Virus in the Alveolar Tissue
of Ozone Exposed Lungs

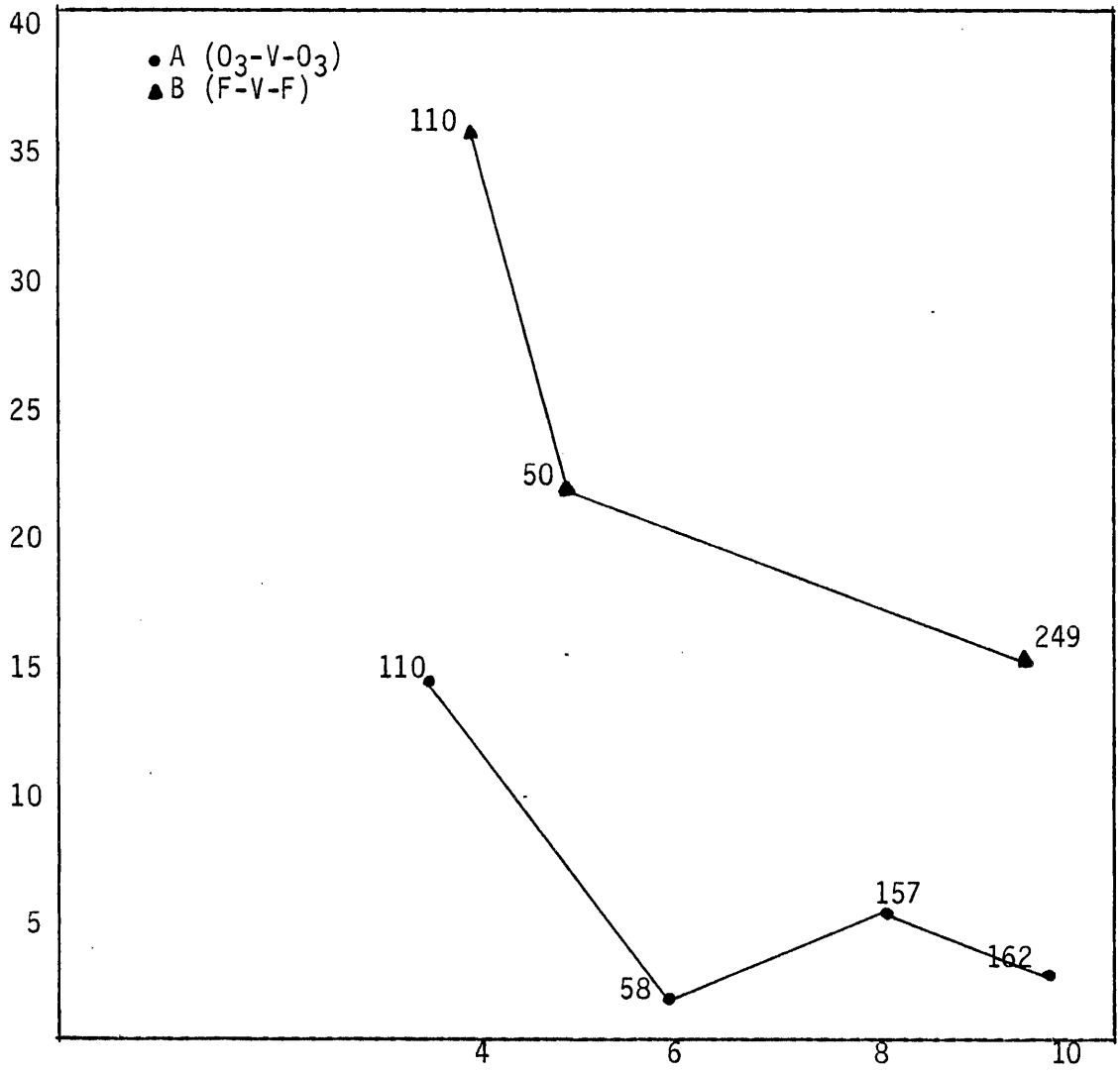


Quantitation of alveolar lung tissue revealing influenza virus by immunofluorescence. Numbers adjacent to data points indicate the number of alveolar fields observed for each determination. O_3, Ozone; V, influenza virus; F, filtered ambient air.

Counts were made as recorded in Figure 4 and Figure 5. In this experiment, the disease severity was not as great in ozone-exposed animals (8%), as that occurring in mice maintained in filtered ambient air (35%). The counts in Figure 4 recorded the numbers of microscopic fields in the alveolar tissue, or gas exchange region of the lung, which showed the presence of influenza virus in infected cells.

The numbers of affected alveolar cells did not vary greatly in mice tested in the two environments. However, Figure 5 indicated that lining epithelial cells of the airways were much less affected by virus replication in animals that had inhaled ozone. This same effect was seen in our previous experiments.

Figure 5
Distribution of Influenza Virus in the Airways of
Ozone Exposed Lungs



Quantitation of airways in lung tissue revealing influenza virus by immunofluorescence. Numbers adjacent to data points indicate the number of airways observed for each determination. O_3, Ozone; V, influenza virus; F, filtered ambient air.

A hypothesis compatible with these results would suggest that an ozone attack on lining cells of the airways produces abnormalities. Epithelial cells of the airways are the primary target cells for an influenza virus attack. However, cells altered by ozone oxidation may no longer serve as normal host cells for virus attachment and replication. The reduced damage from virus propagation in lining epithelium reduces the disease severity and the mortality rate.

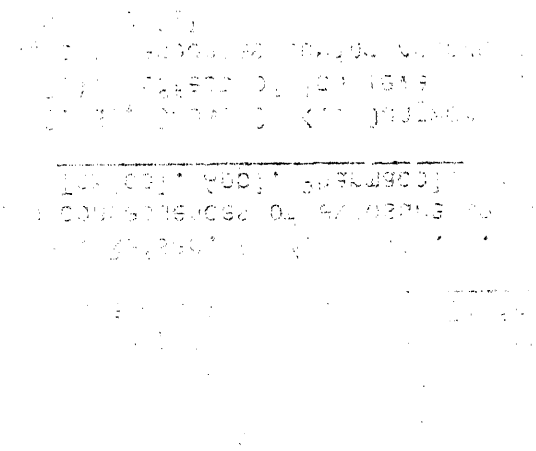
2. Effect of ozone on the influenza virus process when exposures were timed to maximize the edemagenic effects. We have found that the influenza process in mice is less severe in animals that were held in a raised ozone environment before and after exposure to the virus. The parameter that was not tested in those experiments concerned the simultaneous onset of edema from both the oxidant attack by ozone and the virus infection. Chance timing of these events could easily synchronize the two processes for individuals in an exposed population. An experiment was performed to examine the consequences of such events. The design of the study and mortality rates from the experiment are presented in Table 7.

Table 7 - Effect of Maximum Edema from Ozone and Influenza Virus Infection on Mice

	Virus Aerosol	Ozone Exposure**	Maximum Edema	Number of Mice Dead				Dead/ Tested
Days*	0	4	8	10	11	14	16	
Group A - Filtered ambient air	X		X		3		1	4/60
Group B - Ozone Exposed	X	X	X	1		1		2/60

* Days following virus infection.
** Continuous ozone exposure at 0.16 ppm from day 4 through day 20 to Group B only.

Selective timing of the virus infection and ozone exposure did not alter the trend of results obtained in earlier experiments. In those experiments, the mortality rate for mice held in ambient air always exceeded that occurring in ozone exposed groups. That same trend occurred in this experiment, although the magnitude of the difference between test groups was not significant in this study. It was concluded that ozone exposure, even when timed to produce its maximum edemagenic effect, did not increase the severity of influenza over that encountered in animals breathing ambient air. This study concluded the planned experiments to search for possible deleterious health effects from ozone inhalation on the process of influenza virus infection.



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